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Regulation and Role of Sox9 in Cartilage Formation

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ABSTRACT **The HMG-domain transcription factor Sox9 is a known regulator of the type II collagen gene, a major developmentally regulated protein of cartilage. In order to place Sox9 function in skeletogenesis we have investigated the regulation and misexpression of *Sox9* in avian embryos. Application of exogenous BMP2 to chick limbs resulted in upregulation of *Sox9*, concomitant with induction of ectopic cartilage. Ectopic expression of the BMP antagonist Noggin in the limb resulted in loss of *Sox9* expression from the developing digits, indicating that *Sox9* expression during chondrogenesis is BMP dependent. Misexpression of *Sox9* in vivo resulted in ectopic cartilage formation in limbs and in vitro was able to change the aggregation properties of limb mesenchymal cells, suggesting that *Sox9* functions at the level of mesenchymal cell condensation. Misexpression of *Sox9* in dermomyotomal cells, which normally give rise to the axial musculature and dermis, can result in the diversion of these cells from their normal fates towards the cartilage differentiation programme. These cells not only express type II collagen, but also *Pax1*, a marker of ventral fate in the developing somite. This suggests that the cell fate decision to follow the cartilage differentiation pathway is regulated at an early stage by *Sox9*. *Dev Dyn 1999;215: 69–78.*** © 1999 Wiley-Liss, Inc.

Key words: *Sox9*; cartilage; BMPs; Noggin

INTRODUCTION

The ability of cells to contribute to the axial, appendicular and cranial skeletons is dependent upon the acquisition of skeletogenic or chondrogenic potential early in the patterning of the vertebrate body plan. Different parts of the embryonic skeleton are derived from distinct cell lineages. The neural crest gives rise to the branchial arch derivatives of the craniofacial skeleton and intramebranous bones of the skull while the sclerotomal compartment of the somites generate the axial skeleton. The skeletal elements of the limbs are generated from the lateral plate mesoderm and in each case mesenchymal cells aggregate to form condensations and subsequently differentiate to form osteoblasts or chondrocytes.

During endochondral bone formation commitment to a chondrogenic cell type can be detected prior to condensation and differentiation into chondrocytes. For example, mesenchymal cells of the chick limb bud isolated prior to condensation have the ability to form fully differentiated cartilage in vitro (Richman and Tickle, 1992). Moreover, molecular markers of chondrocyte fate, such as type II collagen, can be detected prior to overt chondrogenesis (Nah et al., 1988; Cheah et al., 1991). The processes which bring about this commitment to skeletogenic and chondrogenic cell fate are little understood but may involve transcription factors which commit a population of cells to chondrogenesis.

The Sox gene family is a large group of genes related to the testis determination factor SRY. The family is characterised by the presence of a 79-amino-acid motif (SRY-like HMG-box) which has the properties of a sequence specific DNA binding domain. Sox genes are known to be involved in a number of developmental processes and may act by regulating gene expression via a DNA bending mechanism and/or transcriptional activation/repression (van de Wetering and Clevers, 1992; Dubin and Ostrer, 1994; Giese et al., 1995). It has been suggested that one member of this family of transcription factors, SOX9, is involved in the formation of the axial skeleton on the basis of studies of *Sox-9* gene expression in the mouse (Wright et al., 1995) and its implication in the bone disease campomelic dysplasia (CD) in humans (Foster et al., 1994; Wagner et al., 1994). CD is a disorder of the newborn characterised by congenital bowing and angulation of long bones, together with other skeletal and extraskeletal defects (Mansour et al., 1995). Mutations in single alleles of the *SOX9* gene result in skeletal defects including bowing of the limbs, reduction of the scapulae and pelvis, cranial defects and abnormal spine. In the mouse *Sox-9* is also expressed in mesenchymal condensations of the limb and somite prior to cartilage formation consistent with a primary role in chondrogenesis (Wright et al., 1995). Indeed, recent studies have demonstrated that Sox9 is capable of regulating the type II collagen gene, the primary structural protein of cartilage, both in vivo and in vitro (Bell et al., 1997; Lefebvre et al., 1997).

We report here the cloning of the chick *Sox9* gene and show that it is expressed in cells destined to become

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chondrogenic (the condensations of the sclerotome and limb digits) prior to chondrogenesis. We place Sox9 downstream of BMP signalling in the chondrogenesis pathway by virtue of induction of expression by exogenous BMPs and inhibition by the BMP antagonist Noggin. Using retroviral misexpression we demonstrate that *Sox9* can induce ectopic cartilage in limbs and is able to change the aggregation properties of limb mesenchymal cells in vitro. In addition, retroviral expression of *Sox9* in a non-chondrogenic lineage, the dermomyotome, can induce ectopic cartilage formation, indicating that *Sox9* is an important regulator of chondrogenesis.

RESULTS

The Chicken Sox9 Gene

We identified a 1,479 bp open reading frame (493aa) from the genomic and cDNA sequences corresponding to the mouse and human *Sox9*/*SOX9* coding region and showing 87% amino acid sequence similarity to the predicted mouse and human SOX9 proteins (sequences aligned for best fit). One of the clones contained only the 3' region of the mRNA from which the cDNA was derived. The other clone was found to have an SRY-like HMG-box region most similar to that designated *Sox-9* by Wright et al. (Wright et al., 1993). No similarity to other sequences in GenBank or Swissprot was found outside of the HMG-box. The overlapping chicken *Sox9* cDNA clones constituted a total of 1.2 kb containing a single open reading frame of 430 amino acids (Fig. 1), but comparison with the human and mouse Sox9 sequences suggested the clone was incomplete. Northern blot analysis indicated the presence of a single 3.4-kb embryonic transcript (not shown), suggesting the absence of 1.3 kb from the cDNA. The human *SOX9* mRNA has 1.7-kb long 3'untranslated sequence which was not present in our cDNA clone. A genomic clone was isolated from a chick genomic library constructed in λEMBL3 using the 1.2-kb insert as a hybridisation probe. This yielded a 13-kb fragment which encompased the entire coding region of *Sox9* and included two introns (Fig 1, arrows). We assume, therefore, that the open reading frame isolated in the *Sox9* genomic and cDNA clones corresponds to the coding region of the chicken *Sox9* gene. The genomic organisation of the chick *Sox9* gene is identical to that of the human and mouse genes (Foster et al., 1994; Wagner et al., 1994; Wright et al., 1995), with introns at amino acid 144 and 227 (Fig. 1).

In addition to the SRY-like box the translated ORF of chicken *Sox9* contains a region with approximately 70% sequence identity with the putative C-terminal transcriptional activation domain (Sudbeck et al., 1996; Ng et al., 1997) identified in the human and mouse Sox9 transcription factors (Fig. 1). The chicken *Sox9* cDNA does not share any common regions outside the SRY-like box HMG-box with other cloned Sox genes except for the mouse, rat, alligator and human *Sox9* genes (Wagner et al., 1994; Wright et al., 1995; data not shown).

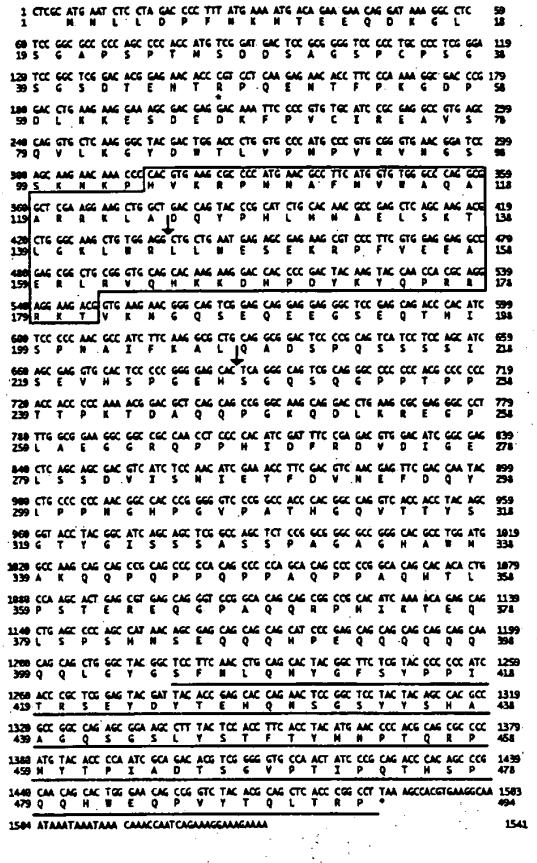

Fig. 1. Two overlapping cDNA's were isolated from a mixed Hamburger and Hamilton stage 14–17 (Hamburger and Hamilton, 1951) embryonic chick cDNA library following probing with a pool of PCR-generated HMG-box sequences (see Materials and Methods). The 5' extent of the cDNA is denoted by an asterisk. The HMG-domain is boxed, introns shown by arrows and the putative activation domain is underlined. The box diagram shows the construct used for retroviral expression using RCAS(A). The amino terminal end of the constuct was derived from a genomic clone (λG16) using PCR primers designed against nucleotides 4–25, introducing an NcoI site around the start codon, and nucleotides 321–339. An internal BamHI site was then removed by site-directed mutagenesis and the 5' clone was ligated to the partial cDNA p20–1 before insertion into RCAS(A).

Misexpression of Sox9 Induces Ectopic Cartilage Formation in Developing Limbs

Whole-mount in situ hybridisation analysis with *Sox9* and type II collagen probes showed that *Sox9* was expressed in all chondrogenic areas of the developing chick embryo (Fig. 2A,B), including the sclerotome, branchial arches, and limbs. Expression of *Sox9* was first observed in the limb bud at stage 22 (Fig. 2A), when transcripts were detected in the mesenchymal

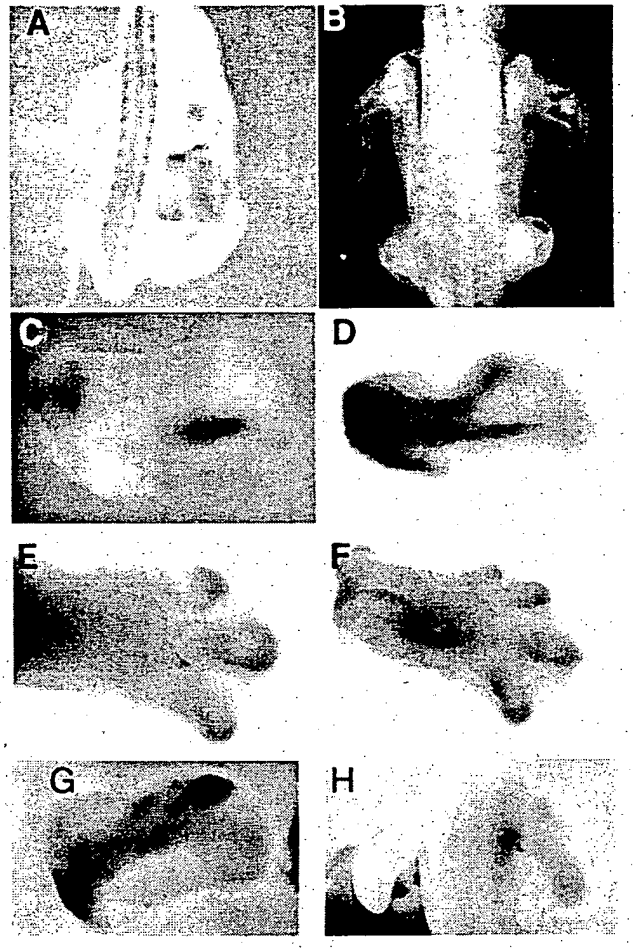

Fig. 2. Whole-mount in situ analysis of *Sox9* and type II collagen during chondrogenesis. Expression of *Sox9* was observed in chondrogenic regions in the branchial arches, frontonasal masses (not shown), somites and limb buds **(A)** in stage 22 embryos. At stage 28 of development *Sox9* was detected in the limbs, developing scapula, prevertebrae and ribs **(B)**. *Sox9* expression was observed in condensing mesenchyme of the forelimb at stage 25 **(C)**. A similar expression pattern was observed for type II collagen at stage 27 in the forelimb **(D)**. *Sox9* was expressed in the condensing mesenchyme at the distal tips of the developing hindlimbs at stage 26 **(E)**, in a very similar pattern to type II collagen **(F)**. Ectopic expression of *Sox9* in the developing forelimb using the RCAS(A)-Sox9 retrovirus results in ectopic induction of type II collagen transcripts throughout the limb bud consistent with the role of *Sox9* as a regulator of type II collagen expression. Whole-mount in situ hybridisation showing extensive spread of RCAS(A)-Sox9 in stage 26 forelimb **(G)**. Induction of ectopic type II collagen in chick forelimb (H) by retroviral expression of *Sox9*. Staining reactions were stopped before the endogenous type II collagen signal became visible.

core of the wing buds. As development proceeds expression becomes more localised within the limb buds, being detected throughout the pre-condensing mesenchyme of the limbs (Fig. 2C, E). Thus, when type II collagen expression starts to mark the developing cartilage of the limbs (Fig 2D), the pattern of *Sox9* expression closely matches that of type II collagen (Fig. 2C, D, E, F). *Sox9* expression thus correlates with areas of newly forming mesenchymal condensations, suggestive of a role in chondrocyte progenitor cell determination.

To investigate the nature of this role in cartilage differentiation we mis-expressed the *Sox9* gene in developing chicken limbs using the RCAS retroviral expression system. The chicken *Sox9* coding region was cloned into RCAS(A) expression vector and concentrated viral supernatants were used to infect stage 10 embryos by injection into the presumptive hindlimb region (n = 50). In addition, viral supernatants were injected into stage 16 forelimbs (n = 40). RCAS(A)-Sox9 infected fibroblasts were also grafted into stage 16 forelimb and hindlimb regions (n = 40). Viral spread was confirmed by whole-mount in situ hybridisation using an RCAS(A) specific riboprobe (Fig. 2G).

Infection of RCAS(A)-Sox9 produced a range of cartilage phenotypes of differing severity (Fig. 3). In "weak" phenotypes there was obvious thickening and shortening of the radius (not shown) or ulna (Fig. 3B) and/or small additional cartilage elements, sometimes protruding from the limb (Fig. 3E, F). The strongest phenotype showed extensive additional cartilage formation and branching of the digit elements (Fig. 3G). Ectopic cartilage was also seen in the scapula and pelvic regins

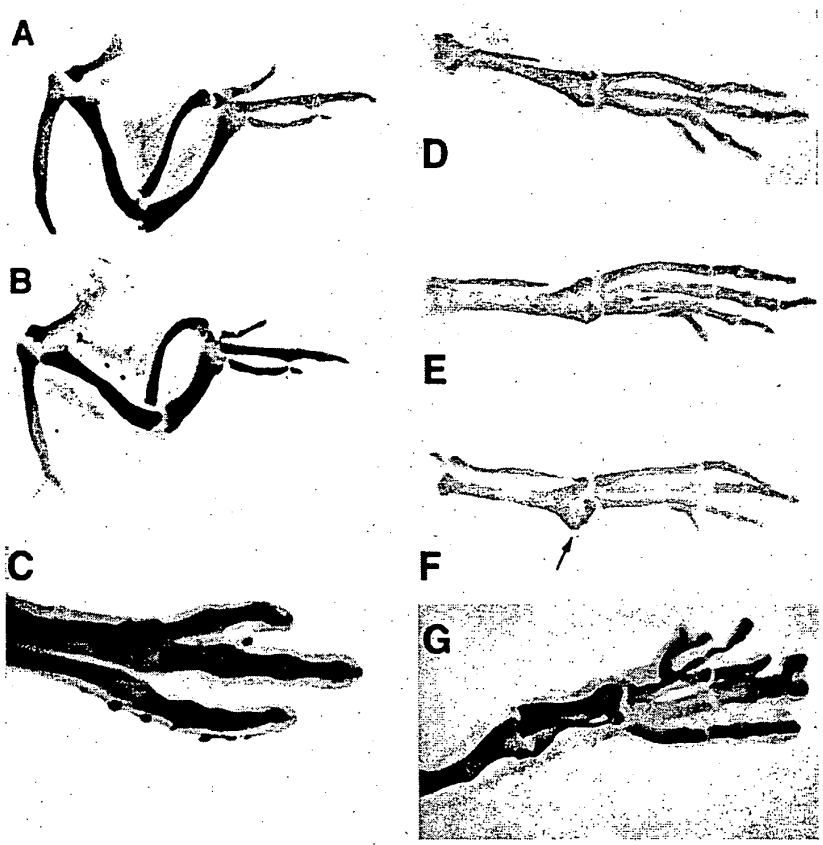

Fig. 3. Ten-day-old RCAS(A)-Sox9 infected limbs show variable phenotypes when compared by alcian blue staining. Forelimbs into which RCAS(A)-Sox9 infected chick embryonic fibroblasts have been grafted at stage 16 show shortening and thickening of the ulna (**B**) when compared to uninfected limbs (**A**). Ectopic interdigital elements and increased cartilage around the joints (**D–F**) were also observed in two grafts (arrows). Thirteen grafts were made using chick embryonic fibroblasts infected with the RCAS-alkaline phosphatase construct, none of which displayed any cartilage phenotype. Injection of RCAS(A)-Sox9 viral supernatant into stage 10 hindlimb regions (approximately fifty injections) resulted in the formation of ectopic cartilage nodules (**C**) in weak phenotypes (five) and extensive branching of the cartilage in the most severe pheotype (**G**).

(not shown). There was no difference in the severity of the phenotype between grafted or infected embryos. The frequency of cartilage phenotypes was low (approximately 10% of infections) with many infected limbs showing no cartilage anomalies. In addition to the induction of ectopic cartilage we also observed induction of type II collagen in the limbs (Fig. 2G, H), consistent with the role of Sox9 as a regulator of type II collagen transcription. Ectopic expression of type II collagen was observed in all infected embryos (n = 12), in constrast to the low frequency of ectopic cartilage formation in limbs ectopically expressing Sox9.

To investigate the basis of the induction of ectopic cartilage by retrovirally expressed *Sox9* we used an in vitro assay for chondrogenesis, the chick limb bud micromass system. Wing bud limb mesenchymal cells were plated at high density and underwent chondrogenic development over a period of four to five days, with precartilaginous nodules beginning to aggregate after 1 day (Fig 4A). Cultures which were infected with RCAS(A)-Sox9 showed a radical difference in aggregation properties. One day after culturing the RCAS(A)-Sox9 infected micromass cultures were completely aggregated (Fig. 4B), suggesting that the retrovirally expressed Sox9 acts by changing the aggregation properties of cells and inducing condensation. In addition, alcian blue staining revealed a large increase in proteoglycan accumulation when compared to cultures infected with an RCAS(A) vector carrying a placental alkaline phosphatase cDNA (Fig 4C, D). We also tested the chondrogenesis-inducing activity of the Sox9 retrovirus with limb mesenchymal cells plated at low density (2×10^7 cells/cm^2). Under these conditions cartilage formation, as judged by alcian blue staining of proteoglycans, was minimal (Fig 4E). Addition of RCAS(A)-Sox9 to these low density micromass cultures

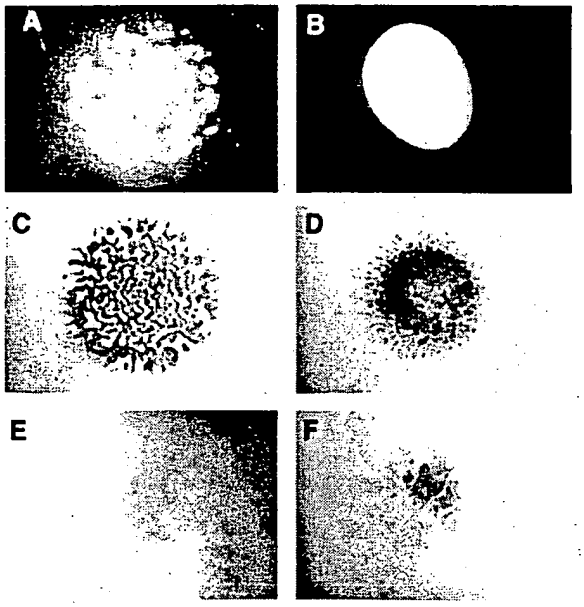

Fig. 4. High density micromass cultures from stage 22–24 chick limbs show increased aggregation after 24 hr (n = 12) with addition of addition of RCAS(A)-Sox9 (B) as compared to cultures (n = 6) where RCAS-alkaline phosphatase retrovirus (A) was added. After 5 days RCAS-alkaline-phosphatase infected limb micromass cultures (C) show reduced cartilage formation (n = 6) as judged by alcian blue staining when compared to RCAS(A)-Sox9 infected cultures (n = 12) (D). Staining of limb micromass cultures plated at low density show only background levels of alcian blue staining (E) indicating that chondrogenesis does not occur under conditions where aggregation is absent. Addition of the *Sox9* expressing retrovirus to these cultures results in staining of cartilage with alcian blue (F) indicating that Sox9 can induce chondrogenesis at low cell densities.

resulted in greatly increased alcian blue staining (Fig 4F), suggesting that *Sox9* can promote cartilage formation when endogenous chondrogensis is minimal.

To investigate the mechanism of *Sox9*-induced chondrogenesis further we studied the expression of the BMP type IB receptor, since this receptor has been shown to play a role in cartilage formation (Zou et al., 1997). BMPRIB expression is localised to prechondrogenic mesenchyme in the developing limb (Fig 5B). Ectopic expression of *Sox9* in the developing limb resulted in an altered pattern of expression of receptor BMPRIB (Fig 5A). In RCAS(A)-Sox9 infected limbs BMPRIB still appeared to be localised to the mesenchymal condensations, although these condensations were branched and appear to be similar in pattern to the ectopic cartilage phenotype in Figure 3G. The BMPRIB receptor did not show any ectopic expression outside of the condensing mesenchyme of the limb indicating that upregulation of receptor BMPRIB expression was not solely due to ectopic *Sox9* expression, but rather to the formation of ectopic condensations in response to ectopic Sox9.

Sox9 is Und r the Control of a BMP-D pendent Pathway

In order to gain insight into the position of Sox9 in the chondrogenic hierarchy we investigated the expression of *Sox9* in relation to the action of the BMP pathway. Implantation of BMP beads into stage 25 and older chick limbs results in ectopic chondrogenesis (Buckland et al., 1998). To test whether this effect correlated with induction of *Sox9* we grafted beads soaked in BMP2 into stage 25 chick limbs and assayed *Sox9* expression 24 hours later. Limbs with implanted BMP2 beads showed ectopic expression of *Sox9* around the bead (Fig. 6A) indicating that BMP can mediate the induction of Sox9. Grafting BMP beads earlier than stage 25 did not result in ectopic chondrogenesis or induction of *Sox9* (Buckland et al., 1998 and data not shown) indicating that chondrogenic competency in response to BMP signalling changes during limb development.

To investigate the role of BMPs in the regulation of *Sox9* further we used a retrovirus expressing the BMP antagonist *Noggin*, RCAS(A)-Noggin (Capdevila and Johnson, 1998). Noggin is known to inhibit the activity of BMP's by binding directly to the proteins (Zimmerman et al., 1996), rendering them unavailable to BMP receptor signalling. We therefore used the RCAS(A)-Noggin retrovirus to inhibit endogenous BMP activity during chondrogenesis in the limb. We infected the limbs at late stages (stage 20) in order to avoid the patterning effects of BMPs during early limb development. Infection with RCAS(A)-Noggin resulted in shortening of the cartilage elements (Capdevila and Johnson, 1998 and data not shown) and loss of some of the digits (Fig. 6C). This loss of the distal phalanges of the digits correlated with a reduction and loss of *Sox9* expression (Fig. 6D) in the newly condensing distal phalangeal regions of the limbs where new cartilage elements are forming. Thus, expression of *Sox9* and subsequent differentiation into cartilage is dependent upon the activity of BMP's in the developing limb.

Ectopic *Sox9* Expression Can Induce Cartilage Formation in Dermomyotomal Progenitors

The cartilage of the axial skeleton is derived from the sclerotome and the expression pattern of *Sox9* in the sclerotome is consistent with a role in the determination of cartilage in the vertebrae. To investigate this role we mis-expressed *Sox9* in the dermomyotomal compartment of the somite. This was possible because the A subtype of the RCAS retroviral vector appears to show a tropic interaction with dermomyotomal cells and spreads specifically in the dermomyotome (Fig. 7C, D). In embryos harvested 48 hours after infection this retroviral expression is associated with ectopic induction of type II collagen transcripts in the dermomyotome (Fig. 7A, E).

In situ hybridisation with the sclerotomal marker *Pax1* in RCAS(A)-Sox9 infected embryos showed that *Pax1* was expressed outside its usual somitic compart-

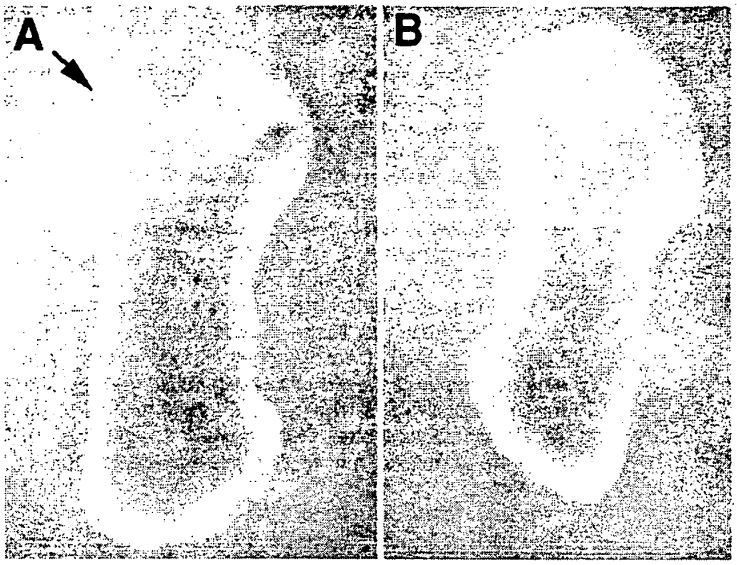

Fig. 5. *BMPRIB* expression in RCAS(A)-Sox9-infected limb 3 days after infection at stage 20. **(A)** Expression of *BMPRIB* remains only in condensations of the limb, but shows ectopic branching (arrow) similar to the cartilage phenotype seen in RCAS(A)-Sox9-infected limbs. **(B)** *BMPRIB* expression in uninfected contralateral limb.

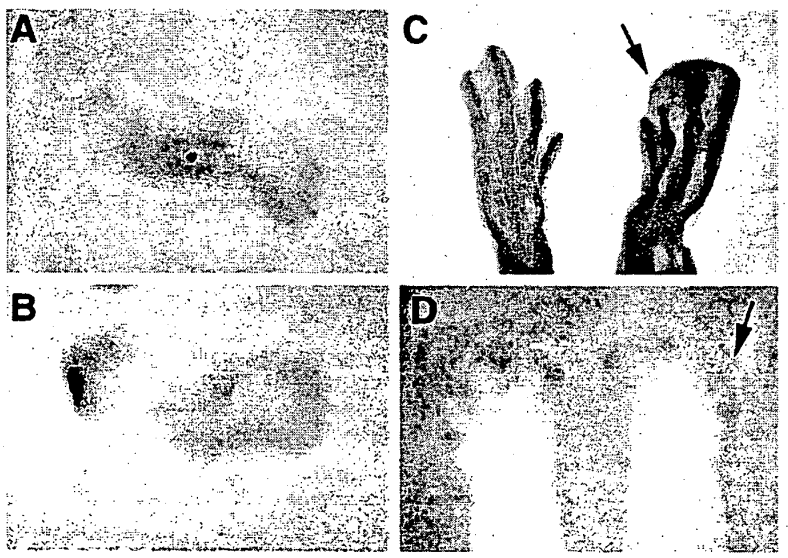

Fig. 6. *Sox9* expression in the limb can be induced by BMP2 and is dependent upon BMP signalling. **(A)** *Sox9* expression 24 hr after implanting BMP2 (100mg/ml) Affigel Blue bead at stage 25 (n = 4). **(B)** *Sox9* expression in the contralateral limb. **(C)** Loss of cartilage from distal phalanges of digits 4 days after infection with RCAS(A)-Noggin (arrow) (n = 12). **(D)** *Sox9* expression is lost in condensing mesenchyme 3 days after infection with RCAS(A)-Noggin at stage 20 (n = 4).

ment in the dermomyotome (Fig. 7B and F). Alcian blue staining of 10-day embryos revealed ectopic cartilage associated with the axial skeleton of RCAS(A)-Sox9 infected embryos (Fig. 7G–J). This cartilage was found in positions dorsal to the vertebrae (Fig. 7H), consistent with this cartilage being derived from the dermomyotome. Thus ectopic expression of Sox9 in the dermomyotome is sufficient to induce the ventral differentiation programme in the dorsal somitic compartment resulting in ectopic cartilage formation. Induction of an early sclerotomal marker, *Pax1*, and a marker of prechondrogenic mesenchyme, type II collagen, indicates that Sox9 is able to implement the ventral differentiation programme in the somite.

DISCUSSION

Sox9 in Axial Skeletogenesis

The ability of Sox9 to regulate type II collagen and the skeletal phenotype of campomelic dysplasia suggested the sclerotomal expression of *Sox9* may be involved in the development of the axial skeleton. In order to test this we ectopically expressed *Sox9* in the dermomyotome and assayed for cartilage differentiation in these cells. Infection of the dermomyotome with

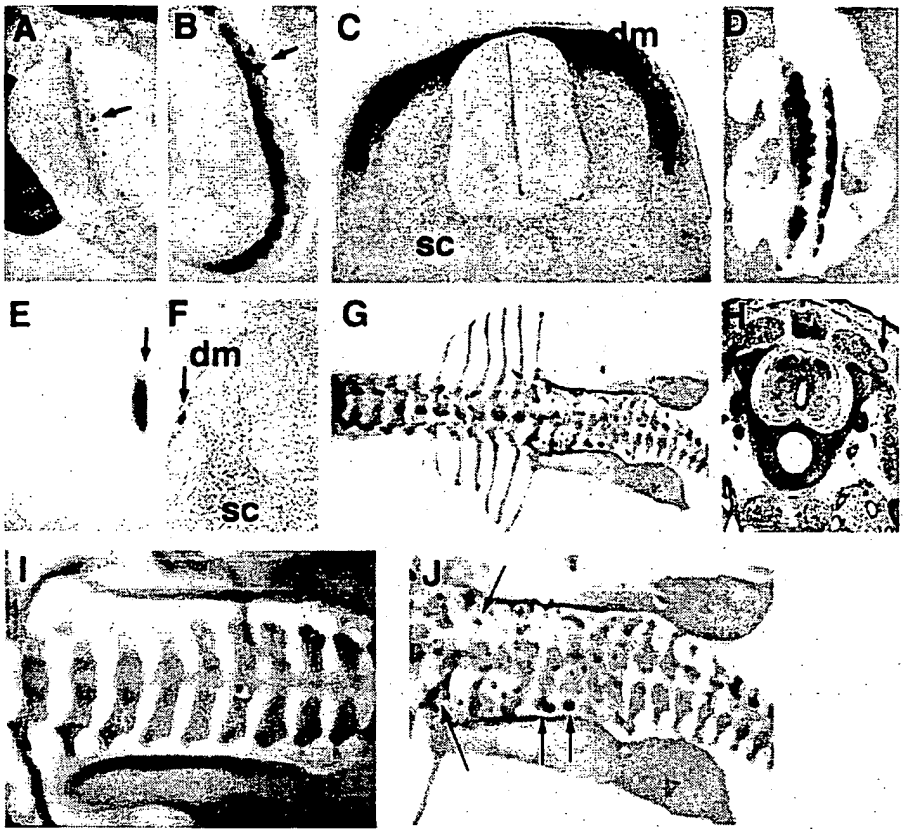

Fig. 7. Infection of dermomyotome cells with RCAS(A)-Sox9 induces the ventral somitic programme. Injection of RCAS(A)-Sox9 retrovirus into the segmental plate at stage 10–12 results in ectopic induction of type II collagen (A) and *Pax1* (B). Viral infection, detected by in situ hybridisation with an RCAS(A) *env* specific probe, is restricted to the dermomyotome (C) and is extensively spread throughout the paraxial mesoderm (D). Vibrotome sections show that ectopic type II collagen (E) and *Pax1* (F) are restricted to the dorsal somitic compartment. Approximately fifty injections were carried out, of which twenty-seven showed ectopic cartilage in the vertebral column (G), scapula, pelvis, or limb (not shown). Alcian blue staining of infected embryos at day 10 shows the formation of ectopic cartilage (arrow) dorsal to the vertebrae (H) in embryos where *Sox9* is ectopically expressed in the dermomyotome. Comparison of hindlimb girdle region of an uninfected (I) with an RCAS(A)-Sox9-infected embryo (J) shows the location of extravertebral cartilage (examples are shown with arrows).

a retroviral expression vector carrying the *Sox9* coding region resulted in the induction of cartilage, a ventral somitic tissue. Along with induction of ectopic cartilage we observed induction of the sclerotomal marker *Pax1* in the dermomyotome, indicating that *Sox9* may have a role both in establishing ventral characteristics of the somite and in directing cartilage differentiation. We are currently investigating the nature of this ventralising activity but it is clear that *Sox9* is able to convert dermomyotomal progenitors from their dermal and muscle fates towards the cartilage differentiation programme. Patterning of the somite into sclerotome and dermomyotome is brought about by the interaction of ventralising signals from the notochord/floor plate complex and dorsalising signals from the surface ectoderm and neural tube (Dietrich et al., 1997). These signals are thought to antagonise each other to promote the formation of compartments within the somite. The failure of many of the RCAS(A)-Sox9 infected dermomyotomal cells to differentiation as cartilage may be due to dorsalising influences from the neural tube and surface ectoderm overcoming the ventralising effects of ectopic *Sox9* expression. When *Sox9* is misexpressed in the dermomyotome a small proportion of cells may escape these dorsalising influences and then undergo cartilage differentiation.

Sox9 and Cartilage Formation

The expression of *Sox9* is consistent with a regulatory role in chondrogenesis. Misexpression of *Sox9* was sufficient to induce condensation both in infected limb buds and in the limb micromass culture system in vitro. A number of cell surface changes such as increased hyaluronan synthesis and binding (Knudson et al., 1995) and the expression of adhesion molecules such as NCAM and N-cadherin (Oberlender and Tuan, 1994; Tavella et al., 1994) have been implicated in the aquisition of cell adhesiveness during condensation of prechondrogenic cells and we are currently investigating whether *Sox9* induced aggregation is mediated by these cell adhesion molecules. The ability of *Sox9* to promote cartilage formation, however, is not confined to its role during aggregation. *Sox9* is clearly a regulator of type II collagen and may also regulate the synthesis of the proteoglycan aggrecan (Sekiya et al., 1997).

The ability to induce aggregation and to direct the expression of later markers of chondrocyte differentiation explains the promotion of ectopic cartilage by the RCAS(A)-Sox9 retrovirus. However, only a small proportion of the cells infected with RCAS(A)-Sox9 go on to form ectopic cartilage. The ability of Sox9 to induce condensation of mesenchymal progenitors is therefore limited by a state of competence or the ability to

differentiate into cartilage subsequently. Clearly then, *Sox9* itself is not sufficient to direct the complete chondrogenic programme. We suggest that other factors are required to act in concert with *Sox9* in order to direct cartilage formation. Recently two other Sox proteins, L-Sox5 and Sox6, have been shown to co-operate with Sox9 in the regulation of the type II collagen enhancer (Lefebvre et al., 1998). Co-expression of L-Sox5, Sox6, and Sox9 in cell lines in which the type II collagen gene is normally silent did not result in activation of the endogenous type II collagen gene. Presumably, therefore, other factors are required for regulation of the *Col2a1* gene. We suggest that non-cartilaginous embryonic cells may be able to react to ectopic expression of *Sox9* alone due to a lack of complete repression of the *Col2a1* enhancer, possibly because a developmental block on chondrogenesis is not fully established in these cells.

We have demonstrated here that *Sox9* is likely to be under the control of a BMP-dependent pathway. Application of BMP soaked beads before stage 25 did not result in ectopic induction of *Sox9* expression. This change in chondrogenic competence to BMPs may result from a change in the pattern of expression of the type I BMP receptors. Transcripts for the type IB BMP receptor (BMPRIB), which has been shown to be necessary and sufficient for cartilage formation, become detectable in the limb by in situ hybridisation at stage 24 of chick development. This roughly corresponds with the time at which application of BMPs to the limb result in ectopic chondrogenesis. Presumably there is a short lag between the onset of BMPRIB expression and chondrogenic competence to respond to BMPs due to the requirement for the proteins to be translated, processed, and assembled before BMP signalling can occur. Inhibition of BMP signalling by misexpressing the BMP antagonist Noggin during digit formation indicates that *Sox9* expression in the early condensing mesenchyme is dependent upon BMP signalling. This loss of expression of *Sox9* in RCAS(A)-Noggin infected limbs correlates with the loss of differentiated cartilage from the distal phalanges of the digits, suggesting that expression of *Sox9* may be required for the formation of the limb cartilage elements. Recent evidence (Harada et al., 1998) indicates that the action of Sox9 may be directly linked to a Smads-dependent BMP pathway, suggesting a direct link between the chondrogenic activity of BMP's and the cartilage differentiation programme via Sox9.

The ability of RCAS(A)-Sox9 to induce cartilage extensively in vitro but much less readily in vivo suggests that the endogenous cartilage patterning programme is in many cases able to overcome the effect of ectopically expressed Sox9. The factors which determine the patterning of the embryonic skeleton are complex and we are currently attempting to identify factors that control which cells will undergo the process of condensation which typifies the earliest identifiable stage in embryonic skeletogenesis.

EXPERIMENTAL PROCEDURES

Isolation of Sox9 cDNA Clones

Two degenerate primers directed against conserved regions of the *SRY*-box of human, mouse, and rabbit *SRY* and *SRY*-related mouse autosomal genes, were synthesised for PCR amplification and cloning of *SRY*-related sequences from chicken genomic DNA (Coriat et al., 1993). The forward primer, designated 8S (5'-ATGGCCC[A/T]GGA[G/A]AACCCCAAGATG-3') was directed against the MA(Q/L)ENPKM motif found in the Sox-gene sub-family that includes *SRY/Sry* and the genes *Sox-1* to *-3*. The reverse primer, designated RG5-L (AGGTCGGGTACCTT[G/A]T[C/T]NGG[A/G]TA-3'), was directed against the YPDYKYRP motif found at the 3' end of the same sub-family of Sox-genes. To allow the resulting PCR fragments to be cloned, each primer contained a terminal recognition site for the restriction enzyme *Hind* III (AAGCTT). The sequence of the cloned PCR fragments was determined and the sub-class containing Sry-related sequences was used to screen a mixed stage 14–17 embryonic λZAPII cDNA library. *Sox9* clones were then isolated by rescuing pBluescript II KS$^+$ (Stratagene) plasmids from individual hybridising plaques.

DNA Sequencing

Double- and single-stranded DNA templates were sequenced in both directions by the dideoxy chain termination method using either the Multi-pol® (Clontech) or Δ*Taq*® Version 2.0 (USB) sequencing kits with α-^{35}S-dATP (Amersham). Unidirectional deletions were made in plasmid-based clones using a protocol based on the Erase-a-base system (Promega) which employed Exonuclease III and S1 nuclease. The 5' sequence of the *Sox9* coding region was determined from a genomic clone isolated from a λGEMSP6T7 chick genomic library. The insert from this genomic clone was subcloned into pBluescript II KS$^+$ (Stratagene) plasmid for sequencing.

Whole-Mount In Situ Hybridisation

Whole-mount in situ hybridisation (Henrique et al., 1995) was carried out with specific probes for the *Sox9*, *Pax1*, *BMPRIB* and type II collagen coding region. A KpnI/ClaI fragment encompassing the *env* region was subcloned from RCAS(A) into pBluescript which was used to generate an antisense probe for the detection of RCAS(A) transcripts. Embryos were harvested from ASA chicken eggs after incubation at 39°C, 30% humidity. For increased sensitivity staining with NBT (Nitro blue tetrazolium, 0.6mg/ml) and X-phos (5-bromo-4-chloro-3-indolyl-phosphate, 0.23mg/ml) (Boehringer) was carried out in 10% polyvinyl alcohol dissolved in NTM (100mM NaCl, 100mM Tris HCl pH 9.5, 50mM $MgCl_2$). Sections (50–100mm) were cut through embryos embedded in gelatin albumin or low melting temperature agarose (FMC) using a Campden Instruments 752M Vibroslice.

Construction of RCAS(A)-Sox9 Retrovirus

Since a full-length cDNA clone for *Sox9* could not be isolated, the full-length coding region had to be assembled from a partial cDNA clone and a genomic clone extending 5′ of the HMG-box. A fragment from the 5′ end of the genomic clone including the putative ATG start codon and introducing an *Nco*I site was generated by PCR. This fragment was cloned into pBluescript by T-tail cloning and an internal *Bam*HI site was removed by site-directed mutagenesis using the ExCite kit (Stratagene). The 5′ end of the *Sox9* coding region was exised as a *Bam*HI fragment and ligated to clone 20–1 thus generating a full length *Sox9* coding sequence (Fig. 1). This reconstituted coding region was cloned into the pCla12 adaptor plasmid, containing the 5′ region of the *src* mRNA to ensure efficient translational initiation. This construct was cloned into the *Cla*I site of RCAS(A). Transfection of the expression construct into O-line chick embryonic fibroblasts (obtained from BBSCRC Institute for Animal Health, Compton, UK) and production and concentration of viral supernatants was carried out according to Morgan and Fekete (Morgan and Fekete, 1996).

Retroviral Infection of Chick Embryos

White Leghorn eggs were used for retroviral infection. Infections were produced either by grafting RCAS(A)-Sox9 infected fibroblasts into stage 16–18 embryos or by injecting concentrated supernatant into stage 10–14 embryos. Grafts were made into the flank of the forelimb or hindlimb regions. Injections (approximately 50 nl) were made into the prospective hindlimb territory or the segmental plate. RCAS(A)-Noggin retrovirus was injected into stage 20 hindlimbs (approximately 50 nl). Embryos were harvested 48 hr after infection to confirm viral infection using an RCAS specific probe (see the In Situ Hybridisation section). Alcian blue staining of embryonic day 10 embryos was carried out according to Tickle (Tickle, 1993).

Limb Bud Mesenchyme Cultures

Limb bud micromass cultures were carried out with stage 24–26 wing buds according to (Daniels et al., 1996) with modifications. Dissociated mesenchymal cells from the proximal half of the limb buds were plated at a density of 4×10^4 or 4×10^5 cells in a 10-µl droplet. Approximately 10^6 retroviral particles were added to the virus treated cultures and the cells allowed to settle for 4 hr before flooding with Hams F12 medium containing 10% foetal calf serum. Cultures were fixed in 10% trichloroacetic acid after 5 days and stained with 0.1% alcian blue (BDH) in 1% HCl, 70% ethanol.

BMP B ad Implantation

Recombinant BMP2 protein (a kind gift of the Genetics Institute, Boston, Mass) was loaded onto Affigel Blue beads (Bio-Rad) as described previously (Tucker et al., 1998) Beads were grafted by making a small incision in the limb with a flame sharpened tungsten needle and pushing the bead in with a blunt tungsten needle.

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